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## **EFA6 activates Arf6 and participates in its targeting to the Flemming body during cytokinesis**

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## ABSTRACT

The small GTPase Arf6 is transiently associated with the ingressing cleavage furrow and subsequently targeted to the Flemming body during cytokinesis, suggesting its activation around the cleavage furrow. Here, we show that EFA6 (exchange factor for Arf6) localizes on the cleavage furrow through its PH domain. Time-lapse analysis showed that both EFA6 and Arf6 are transiently localized around the ingressing cleavage furrow, but only Arf6 is subsequently targeted to the Flemming body. Expression of an EFA6 mutant suppresses Arf6 recruitment onto the Flemming body. These results suggest that EFA6 participates in activation of Arf6 around the cleavage furrow during cytokinesis.

## Keywords

Arf6; cleavage furrow; cytokinesis; EFA6; Flemming body; PH domain

## Abbreviations

Arf, ADP-ribosylation factor; EFA6, exchange factor for Arf6; GEF, guanine nucleotide exchange factor; MKLP1, mitotic kinesin-like protein 1

## Highlights

- Arf6 is required for faithful completion of cytokinesis.
- Arf6 is activated by EFA6 in the cleavage furrow region during cytokinesis.
- Activated Arf6 is targeted to the Flemming body through interacting with MKLP1.

## 1. INTRODUCTION

The Arf (ADP-ribosylation factor) small GTPases participate in various membrane trafficking events. In mammals, there are six Arf isoforms and many Arf-like proteins [1]. The Arf proteins can be grouped into three classes based on sequence similarity: class I, Arf1–Arf3 (humans lack Arf2); class II, Arf4 and Arf5; and class III, Arf6. Among them, Arf1 and Arf6 are the best characterized to date. Arf1 triggers the formation of coated carrier vesicles from the Golgi apparatus and endosomes. Arf6 localizes to the plasma membrane and the endosomal system, where it regulates endosome recycling and remodeling of actin cytoskeleton and membranes [2, 3]. In addition to these roles in interphase cells, Arf6 is required for the final phase of cytokinesis [4–7].

We have recently shown that Arf6 is transiently localized to the ingressing cleavage furrow region, and subsequently recruited to the Flemming body during late cytokinesis [6, 8]. The Flemming body is a dense structure in the overlapping region of antiparallel microtubule bundles of the central spindle; it serves as a scaffold for a wide variety of structural and regulatory proteins required for completion of cytokinesis, including those implicated in membrane trafficking [9, 10]. Furthermore, we have shown that GTP-bound, but not GDP-bound, Arf6 is recruited to the Flemming body through interaction with mitotic kinesin-like protein 1 (MKLP1) [6]; MKLP1 constitutes the centralspindlin complex along with MgcRacGAP/Cyk4, localizes to the Flemming body and plays an essential role in cytokinesis [11, 12]. On the other hand, Joseph *et al.* have recently proposed that Arf6 stabilizes the centralspindlin complex by competing with the 14-3-3 protein [7].

The recruitment of Arf6 to the Flemming body in late cytokinesis phase involves its local and timely activation. Like other GTPases, Arfs cycle between a GDP-bound inactive state and a GTP-bound active state; exchange of bound GDP for GTP is catalyzed by guanine-nucleotide exchange factors (GEFs), while hydrolysis of bound GTP to GDP is promoted by GTPase-activating proteins [2]. All known Arf-GEFs contain a Sec7 catalytic domain. Fifteen Arf-GEFs encoded in the human genome can be divided into five subfamilies based on sequence similarity and domain organization [13–15]. Among them, members of the cytohesin, EFA6 (exchange factor for Arf6), and BRAG (brefeldin A –resistant Arf-GEF) subfamilies have been implicated in Arf6 activation [13].

In this study, we systematically examined these Arf-GEFs, and found that EFA6 becomes localized to the cleavage furrow and is involved in local activation of Arf6 during cytokinesis. Unlike Arf6, however, EFA6 is not targeted to the Flemming body.

## 2. MATERIALS AND METHODS

## 2.1. Antibodies and reagents

Sources of antibodies reagents are as follows: polyclonal rabbit anti-Arf6, provided by Yasunori Kanaho (University of Tsukuba, Japan); monoclonal rat anti- $\alpha$ -tubulin, Abcam; monoclonal mouse anti- $\beta$ -tubulin (KMX-1), Millipore; monoclonal rat anti-HA (3F10), Roche Applied Science; AlexaFluor-conjugated secondary antibodies and phalloidin, Molecular Probes.

## 2.2. Plasmids

Human EFA6A cDNA (BC142643) was obtained from OPEN BIOSYSTEMS, and other EFA6 cDNAs were obtained by RT-PCR of human (for EFA6B) or mouse (for EFA6C and EFA6D) total RNAs. cDNAs for cytohesins were obtained by RT-PCR of mouse mRNAs; those for BRAGs were obtained by RT-PCR of mouse (for BRAG1 and BRAG2) or rat (for BRAG3) mRNAs. The cDNAs were subcloned into pEGFP-C1 or pEGFP-C3 (Invitrogen), or pcDNA3-mCherry-C (provided by Roger Tsien, UCSD). An expression vector for Arf6-HA and Arf6-EGFP were described previously [6]. Site-directed mutagenesis were performed using a QuikChange Mutagenesis Kit (Agilent Technologies).

## 2.3. RT-PCR analysis

Total RNAs of HeLa cells were reverse-transcribed to cDNAs using random hexamers and a SuperScript III First-Strand Synthesis System (Invitrogen), and amplified with rTaq DNA Polymerase (TOYOBO) using specific primers (Table S1). The PCR products were confirmed by agarose gel electrophoresis. For EFA6A and EFA6C, the PCR products were extracted from the gel and confirmed by sequence analysis.

## 2.4. Cell culture, DNA transfection, and immunofluorescence analysis

Culture of HeLa cells, transfection of expression plasmids, and immunofluorescence analysis were performed as described previously [16], except for detection of endogenous Arf6. For Arf6, cells were fixed with 10% TCA for 15 min on ice, permeabilized with 0.1% Triton X-100 in PBS for 5 min as described previously [6, 8].

## 2.5. Time-lapse imaging

Cells were seeded on a collagen-coated 35-mm glass bottom dish (Mat Tek) and grown to 30% confluence. Cells were then transfected with expression plasmids for Arf6-EGFP and mCherry-EFA6A, and after 24 hr placed on the microscope stage that had been pre-warmed at 37°C. The cells were observed with an Axiovert 200M microscope (Carl Zeiss) equipped with EMCCD C9100-02 (Hamamatsu Photonics). Images were acquired sequentially every 1 min and analyzed using MetaMorph imaging software (Molecular Devices).

## 2.6. Pulldown assay for active Arf6

GEF activity of EFA6 towards Arf6 in cells was determined as previously described for detection of

Arf-GEF activity of BIG2 [16, 17]. Briefly, lysates were prepared from HeLa cells transfected with an Arf6-HA vector together with either an EGFP-EFA6A or a control EGFP vector, and subjected to pulldown using a GST fusion of the GGA1(GAT) domain pre-bound to glutathione-Sepharose beads (GE Healthcare Bioscience). The bound proteins were then processed for immunoblot analysis using an anti-HA antibody.

### 3. RESULTS

#### 3.1. Transient localization of EFA6 to the cleavage furrow

To find candidate(s) for Arf-GEFs involved in local activation of Arf6 during cytokinesis, we first systematically examined localization of Arf-GEFs. The GBF/BIG subfamily of high-molecular weight Arf-GEFs are predominantly localized to the Golgi apparatus and endosomes, and activate class I and II Arfs, whereas the cytohesin, EFA6 and BRAG subfamilies are primarily localized on the plasma membrane and can activate Arf6 [13, 15]. Therefore, we examined localization of members of the latter subfamilies. When expressed as mCherry-tagged proteins in HeLa cells, in interphase all members of the EFA6 subfamily are associated with the plasma membrane, in particular with membrane ruffling regions (Fig. 1A) as reported previously for EFA6A [18]. In cells undergoing cytokinesis, mCherry-EFA6A and -EFA6C, but not mCherry-EFA6B or -EFA6D, are prominently found around the ingressing cleavage furrow (Fig. 1B). Comparing cells stained for the contractile ring with phalloidin and for the central spindle with anti- $\beta$ -tubulin indicated that EFA6A becomes transiently localized to the cleavage furrow region, but not to the Flemming body (Fig. 1, C-C'''). Essentially the same results were obtained for EFA6C (data not shown). In contrast to EFA6, none of the cytohesin or BRAG subfamily members exhibits significant localization to the cleavage furrow or the Flemming body during cytokinesis (Fig. S1).

All members of the EFA6 subfamily have a Sec7 catalytic domain, a pleckstrin homology (PH) domain, and a coiled-region in their C-terminal half (see Fig. 2A) [19, 20]. Previous studies revealed that mRNAs encoding EFA6A and EFA6C are expressed primarily in the brain, EFA6B mRNA in non-neural tissues, and EFA6D mRNA in all tissues examined [19-21]. No studies have yet examined the expression of these genes in cultured cell lines. In order to establish the physiological relevance of the localization of EFA6A and EFA6C to the cleavage furrow region in HeLa cells, we performed RT-PCR analysis and found that HeLa cells express mRNAs encoding all EFA6 subfamily members (Fig. S2). Since EFA6A has been the most extensively studied, we hereafter focus on EFA6A. We examined exogenous fluorescent protein-tagged EFA6A in the following experiments, because we failed to raise antibodies that can detect endogenous EFA6A or any other EFA6 member in immunoblot and immunofluorescence analyses (data not shown).

### 3.2. PH domain-dependent localization of EFA6 to the cleavage furrow

Previous studies showed that association of EFA6A with the plasma membrane is mediated primarily by its PH domain, which is located C-terminal to the Sec7 domain (see Fig. 2A) and was shown to interact with  $\text{PtdIns}(4,5)\text{P}_2$  [22]. We therefore examined whether the PH domain is required for the cleavage furrow localization of EFA6A. First, we attempted to determine whether the EFA6A PH domain alone was sufficient for localization to the furrow region; this attempt was unsuccessful, however, because expression of the PH domain alone caused death of almost all of the transfected cells for an unknown reason (data not shown). In contrast, expression of a construct encompassing the N- or C-terminal region in addition to the PH domain (Fig. 2A) does not detectably affect cell viability. Both the EFA6A(N+PH) and EFA6A(PH+C) constructs are associated with the cleavage furrow (Fig. 2, C-C'' and D-D'') like EFA6A(WT) (Fig. 2, B-B''). Furthermore, mutation of consecutive Arg and Lys residues in the PH domain (Arg756 and Lys766 in human EFA6A, Fig. 2A), which are conserved in all EFA6 members [19] and were shown to be critical for association of the PH domain with the plasma membrane [22], abolishes the cleavage furrow localization of the full-length EFA6A construct (Fig. 2, E-E''). Taking into account previous reports showing that  $\text{PtdIns}(4,5)\text{P}_2$  is concentrated at the cleavage furrow [23-25], these observations indicate that the PH domain is responsible for association of EFA6A with the cleavage furrow membrane.

### 3.3. Transient colocalization of EFA6 and Arf6 at the cleavage furrow

The above observations suggest that EFA6 is a prime candidate for an Arf-GEF involved in activation of Arf6 around the ingressing cleavage furrow. To explore this idea, we compared localization of EGFP-EFA6A with that of endogenous Arf6 during cytokinesis. In early cytokinesis phase, both EFA6A and Arf6 are found in the cleavage furrow region (Fig. 3, A-A''). As cytokinesis proceeds, Arf6 becomes concentrated at the Flemming body as we have recently reported [6], while EFA6A appears to be gradually delocalized from the cleavage furrow (Fig. 3, B-B'' and C-C''). To demonstrate the behavioral difference between EFA6A and Arf6 during cytokinesis, we then performed time-lapse analysis of cells expressing mCherry-EFA6A and Arf6-EGFP (Fig. 3D and Video S1). In metaphase, when the cell is spherical, both mCherry-EFA6A and Arf6-EGFP are distributed uniformly on the plasma membrane as well as throughout the cytoplasm (panel for 0:00). Both proteins are subsequently concentrated at patches in the equatorial region at the onset of cytokinesis (0:08), and become distributed in the equatorial region concurrent with cleavage furrow ingression (0:13-0:29). In later cytokinesis phase, Arf6-EGFP signals are markedly concentrated at the Flemming body, while mCherry-EFA6A signals fade away from the cleavage furrow and become uniformly distributed throughout the plasma membrane (0:44-1:24). After abscission of the intercellular bridge, Arf6-EGFP is incorporated into one of the daughter cells as a Flemming body remnant

as well as localized to punctate structures beneath the plasma membrane (2:44), as we previously reported [6]. These observations make it likely that Arf6 is activated by EFA6 at the cleavage furrow and in turn recruited to the Flemming body.

### 3.4. Exogenously expressed EFA6 mutant prevents Arf6 from Flemming body localization

To confirm that local activation of Arf6 by EFA6 is required for its localization to the Flemming body, we exploited an EFA6 mutant constructed on the basis of structural data obtained from other Arf-GEFs. All Arf-GEFs have an invariant Glu residue in their Sec7 catalytic domain (for example, Glu156 in cytohesin-2/ARNO); the ‘glutamate finger’ plays a pivotal role in guanine nucleotide exchange on Arfs, and Arf-GEF mutants, where the Glu residue is replaced with Lys, inhibits GDP release from Arfs [26, 27]. We therefore constructed a Glu-to-Lys mutant (E621K) of EFA6A, and asked whether its expression affects Arf6 localization.

We first confirmed that the EFA6A(E621K) mutant lacks GEF activity by performing a pulldown assay with a GST fusion of the GGA1-GAT domain, which detects GTP-bound, but not GDP-bound, Arfs [16, 17]. As shown in Fig. 4A, coexpression of EGFP-EFA6A(WT) in HeLa cells significantly increases the amount of Arf6-HA pulled down with GST-GGA1-GAT as compared with the control, indicating that coexpressed EFA6A can activate Arf6 in the cell. In contrast, EGFP-EFA6A(E621K) expression does not increase the amount of GTP-bound Arf6.

We next examined whether the catalytically inactive EFA6A mutant has any effect on Arf6 localization. In cells expressing exogenous EGFP-EFA6A(WT), endogenous Arf6 is able to localize to the Flemming body (Fig. 4, B-B’'), as in cells without exogenous EFA6 expression [6]. In striking contrast, in cells expressing EGFP-EFA6A(E621K), Arf6 no longer associates with the cleavage furrow nor the Flemming body in late cytokinesis phase (Fig. 4, C-C’'' and F), indicating that Arf6 is locally activated by EFA6 and becomes transiently associated with the furrow membrane, and is in turn recruited onto the Flemming body through interacting with MKLP1 as described previously [6]. To examine whether the observed effect of EFA6A(E621K) is dependent on its cleavage furrow localization, we then exploited mutations in two conserved basic residues in the PH domain (R765E/K766E). The EFA6A(R765E/K766E) mutant is not localized on the plasma membrane in interphase (data not shown) nor to the cleavage furrow region during cytokinesis (Fig. 4, D-D’'', also see Fig. 2E); furthermore, it does not affect the Flemming body localization of Arf6 (Fig. 4, D-D’''). Introduction of the R765E/K766E mutations into the EFA6A(E621K) mutant also abolished its localization to the cleavage furrow (Fig. 4, E-E’''). Notably, as compared with the EFA6A(E621K) mutant (Fig. 4, C-C’''), exogenous EFA6A(E621K/R765E/K766E) expression does not have a drastic effect on the Flemming body localization of Arf6 in late cytokinesis phase



(Fig. 4, E-E''' and F). Thus, these results indicate that the dominant effect of the EFA6A(E621K) mutant on the Arf6 localization depends on its ability to associate with the plasma membrane, probably at the cleavage furrow, through its PH domain.

#### 4. DISCUSSION

We have recently shown that Arf6 is transiently associated with the cleavage furrow, and subsequently recruited to the Flemming body in its GTP-bound state via interaction with MKLP1/KIF23; at the furrow, Arf6 plays a role in successful completion of cytokinesis [6, 7]. The spatiotemporal change of Arf6 localization suggests that it is activated at the cleavage furrow during cytokinesis. Here, we extended these observations by demonstrating that EFA6 becomes localized at the cleavage furrow membrane during cytokinesis (Fig. 1). Arf6 also becomes localized to the cleavage furrow concomitant with the concentration of EFA6 there, suggesting that Arf6 is activated by EFA6 at the furrow (see Fig. 5). Arf6 is in turn targeted to the Flemming body. After abscission, Arf6 is incorporated into one of the daughter cells as a Flemming body remnant along with MKLP1/KIF23 as we recently reported [6], while EFA6 becomes once again distributed throughout the plasma membrane (Fig. 3C and Video S1). Furthermore, we showed that the EFA6A(E621K) mutant, but not EFA6A(E621K/R765E/K766E), dominantly inhibits Arf6 localization to the Flemming body. These observations suggest that Arf6 activation by EFA6 at the cleavage furrow occurs in a manner dependent on EFA6 association with the furrow membrane through its PH domain (Fig. 4). We have also attempted to show involvement of EFA6 in Arf6 activation and cytokinesis by RNAi approach. Because EFA6 isoforms probably have a redundant role, we have attempted simultaneous knockdown of multiple EFA6 isoforms; the attempt, however, has so far been unsuccessful, for lack of specific antibodies against EFA6 isoforms to confirm efficient depletion of the EFA6 proteins. This is a future issue to be addressed.

We propose a functional model (Fig. 5) integrating our data presented in this study with previously published works. Our model suggests that the EFA6 recruitment to the cytoplasmic surface of the cleavage furrow membrane determines the timing of Arf6 activation and its subsequent targeting to the Flemming body via interaction with MKLP1/KIF23, although our results do not exclude a possibility that other Arf-GEF(s) also contribute to the Arf6 activation. The association of EFA6A with the cleavage furrow is abolished by a PH domain mutation (Fig. 4D), which also blocks its association with the plasma membrane [22]. Since PtdIns(4,5)P<sub>2</sub> and PtdIns4P 5-kinase, which catalyzes formation of PtdIns(4,5)P<sub>2</sub> from PtdIns(4)P, are concentrated around the cleavage furrow [23-25], the local generation of PtdIns(4,5)P<sub>2</sub> may determine recruitment of EFA6 to the cleavage furrow membrane and in turn local activation of Arf6.

Furthermore, we previously showed that Arf6 can activate PtdIns4P 5-kinase [28]. It is therefore tempting to speculate that a local, small increase in the active Arf6 level may trigger a subsequent amplification in the signal via local activation of PtdIns4P 5-kinase and a consequent increase in the PtdIns(4,5)P<sub>2</sub> level. Future studies should address the link between local activation of Arf6 and regulation of phosphoinositide metabolism.

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## FIGURE LEGENDS

### Fig. 1. Localization of EFA6 isoforms in interphase and during cytokinesis

(A and B) HeLa cells expressing mCherry-EFA6A (a-a''), -EFA6B (b-b''), -EFA6C (c-c''), or -EFA6C (d-d'') were subjected to staining for  $\beta$ -tubulin (a'-d'). Representative images of cells in interphase (A) and in late cytokinesis phase (B) are shown. (C) HeLa cells expressing EGFP-EFA6A were subjected to staining with AlexaFluor555-phalloidin and with anti- $\beta$ -tubulin antibody followed by AlexaFluor647-conjugated secondary antibody.

### Fig. 2. Localization of various EFA6A constructs during cytokinesis

(A) Schematic representation of the domain organization of EFA6A. The relative positions of E621, R765, and K766 residues are indicated. The EFA6A(N+PH) and EFA6A(PH+C) constructs encompass residues 1-901 and 730-1024, respectively. (B-E) HeLa cells expressing EGFP-EFA6A(WT) (B-B''), -EFA6A(N+PH) (C-C''), -EFA6A(PH+C) (D-D''), or -EFA6A(R765E/K766E) (E-E'') were subjected to staining for  $\beta$ -tubulin (B'-E'). Representative images of cells in late cytokinesis are shown.

### Fig. 3. Comparison of localization of EFA6A and Arf6 during cytokinesis

(A-C) HeLa cells expressing EGFP-EFA6A were subjected to staining for Arf6 (A'-C') and  $\beta$ -tubulin (A''-C''). Images of EGFP-EFA6A (A-C) are arranged from early to late cytokinesis phases in temporal order. (D) HeLa cells expressing mCherry-EFA6A and Arf6-EGFP were subjected to time-lapse recording. An image sequence from supplemental Video S1 is shown.

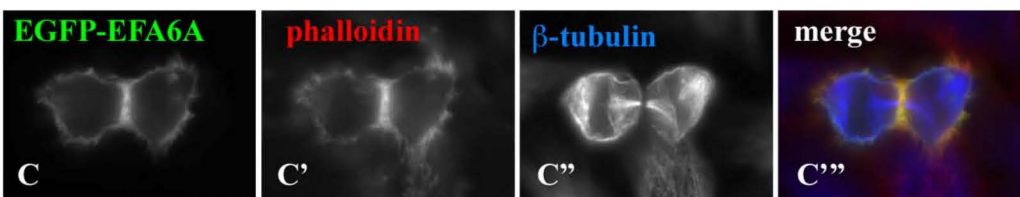
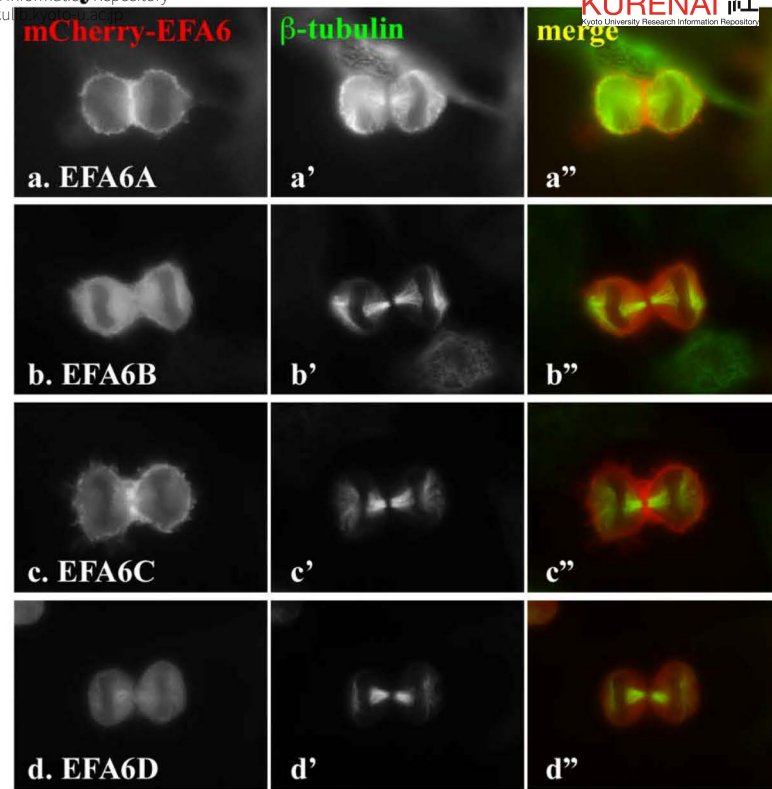
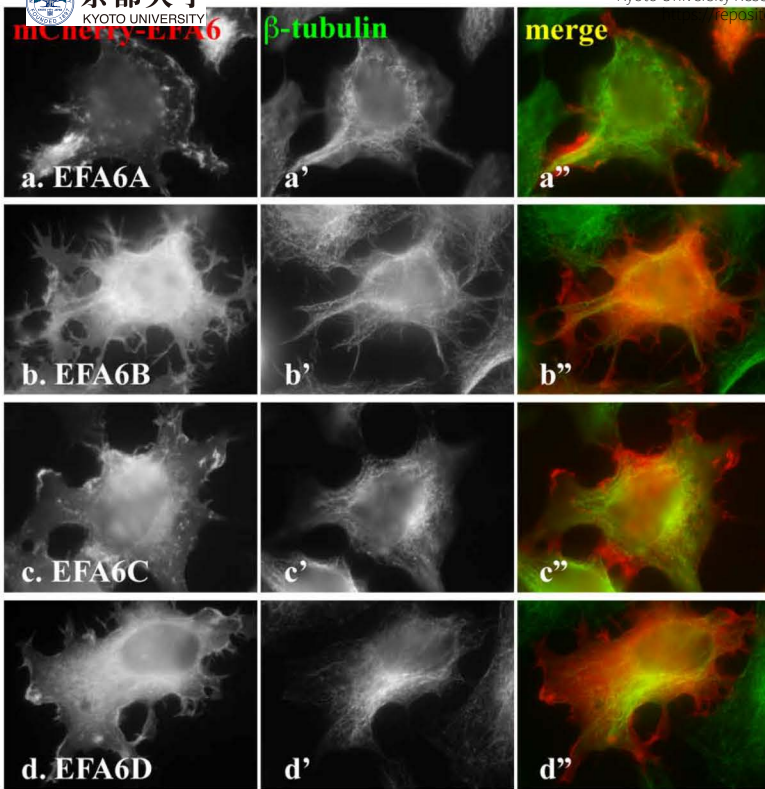
### Fig. 4. Exogenously expressed EFA6 mutant abolishes the Flemming body localization of Arf6

(A) The EFA6A(E621K) mutant lacks GEF activity towards Arf6. Lysates prepared from HeLa cells co-expressing Arf6-HA and either EGFP-EFA6A(WT) or EFA6A(E621K) were subjected to pulldown using GST-GGA1(GAT) and immunoblot analysis using anti-HA antibody. (B-F) Exogenous expression of EFA6A(E621K) abolishes the Flemming body localization of Arf6 in a manner dependent on cleavage furrow association via its PH domain. HeLa cells expressing EGFP-EFA6A(WT) (B), -EFA6A(E621K) (C), -EFA6A(R765E/K766E) (D), or -EFA6A(E631K/R765E/K766E) (E) were subjected to staining for Arf6 and  $\beta$ -tubulin. Representative images of cells in late cytokinesis are shown. (F) The cells in late cytokinesis in the experiments shown in (B)-(E) were classified as with or without endogenous Arf6 signals at the Flemming body. Percentages of cells with the Arf6 signals at the Flemming body are expressed as bar graphs. Note that, because not only the expression efficiency of the EFA6A(R765E/K766E) mutant but also

the proportion of EFA6A(R765E/K766E)-expressing cells in cytokinesis was extremely low, we could examine only a small number of EFA6A(R765E/K766E)-expressing cells, as compared with cells expressing other EFA6A(WT) constructs.

**Fig. 5. A model for Arf6 activation by EFA6 and subsequent targeting to the Flemming body**

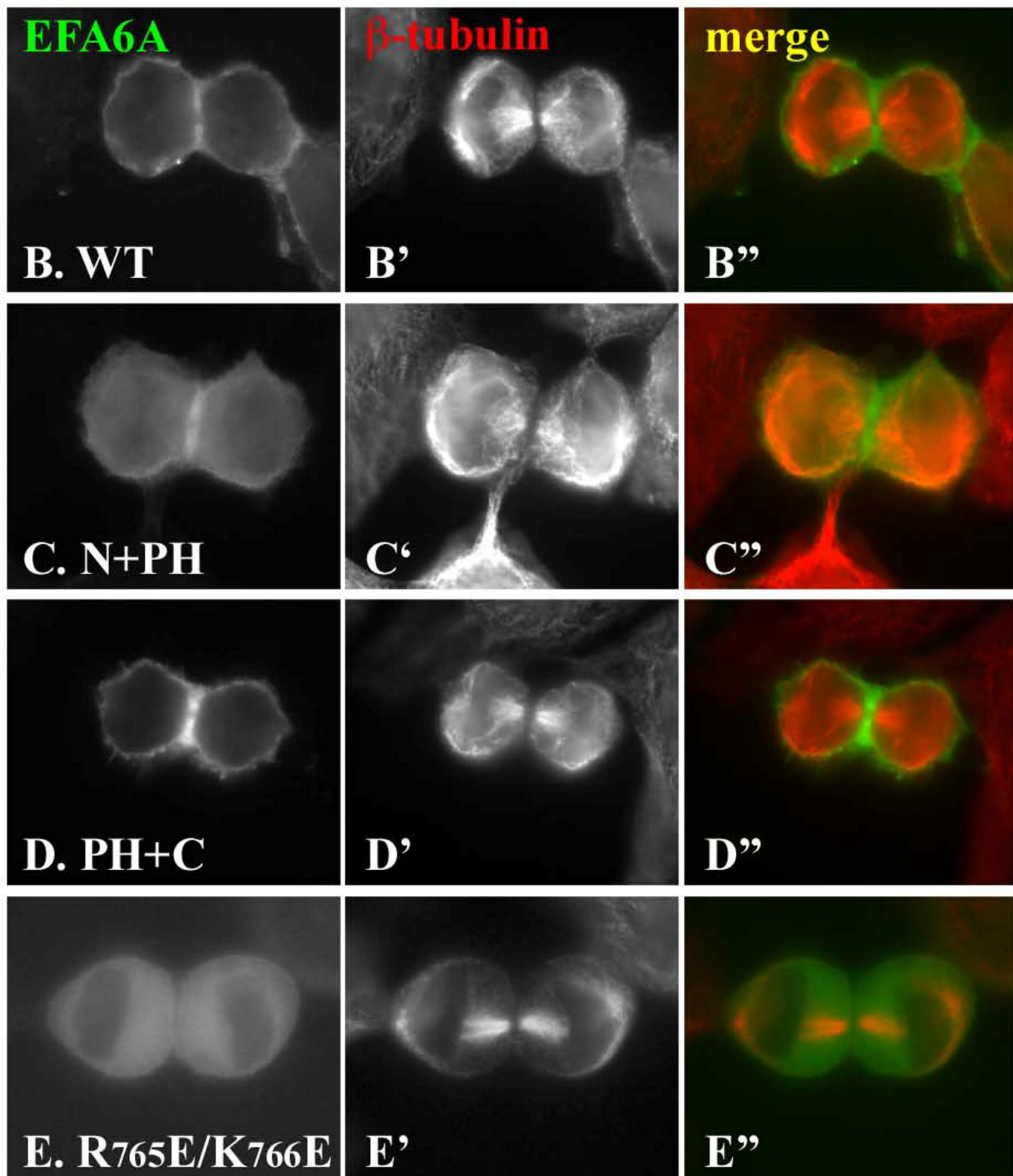
EFA6 is concentrated to the cleavage furrow membrane by interacting with PtdIns(4,5)P<sub>2</sub> via its PH domain, and promotes exchange of GDP bound to Arf6 for GTP. The GTP-bound Arf6 is then recruited to the Flemming body by interacting with preexisting MKLP1.



**Fig. 1: Ueda et al.**

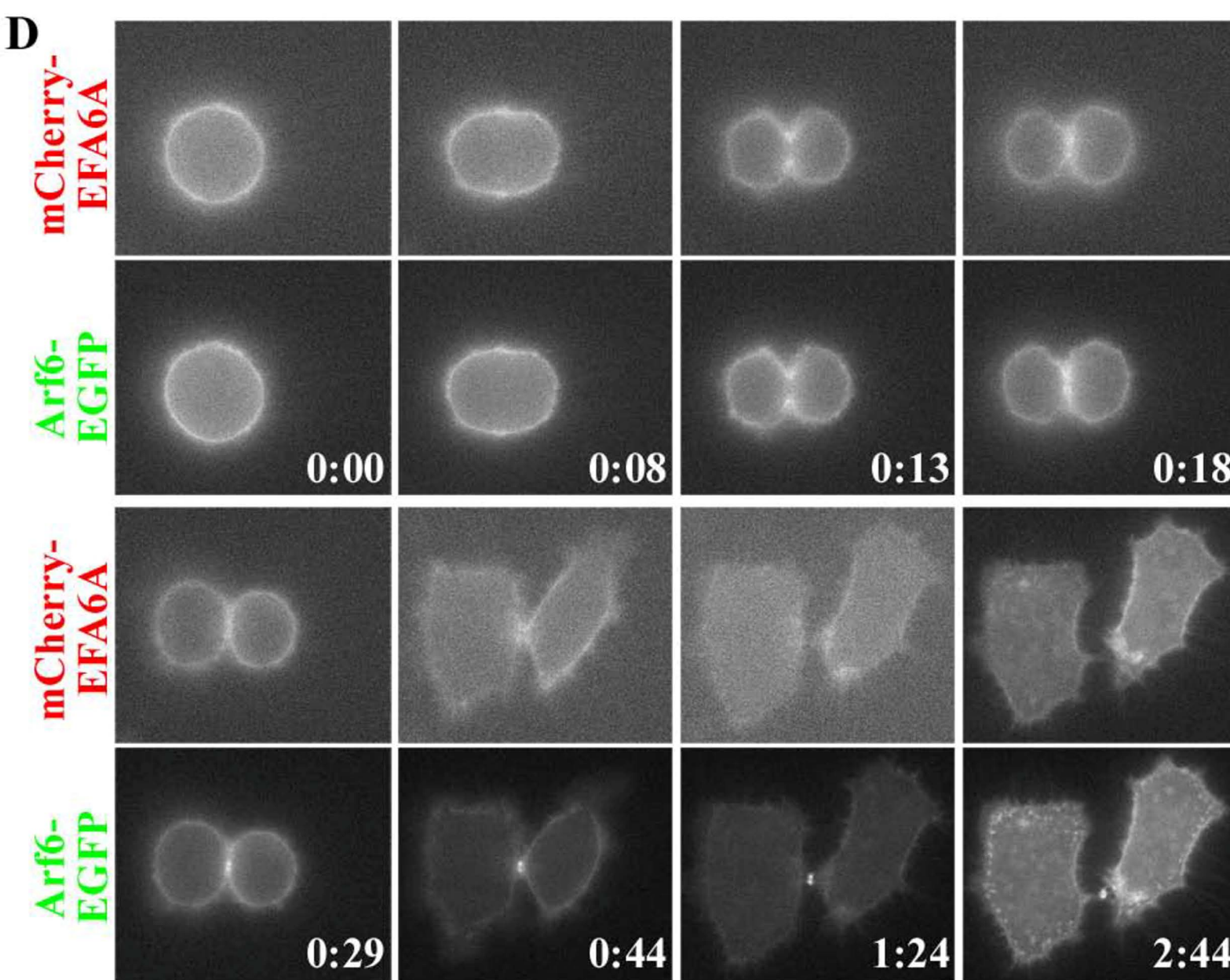
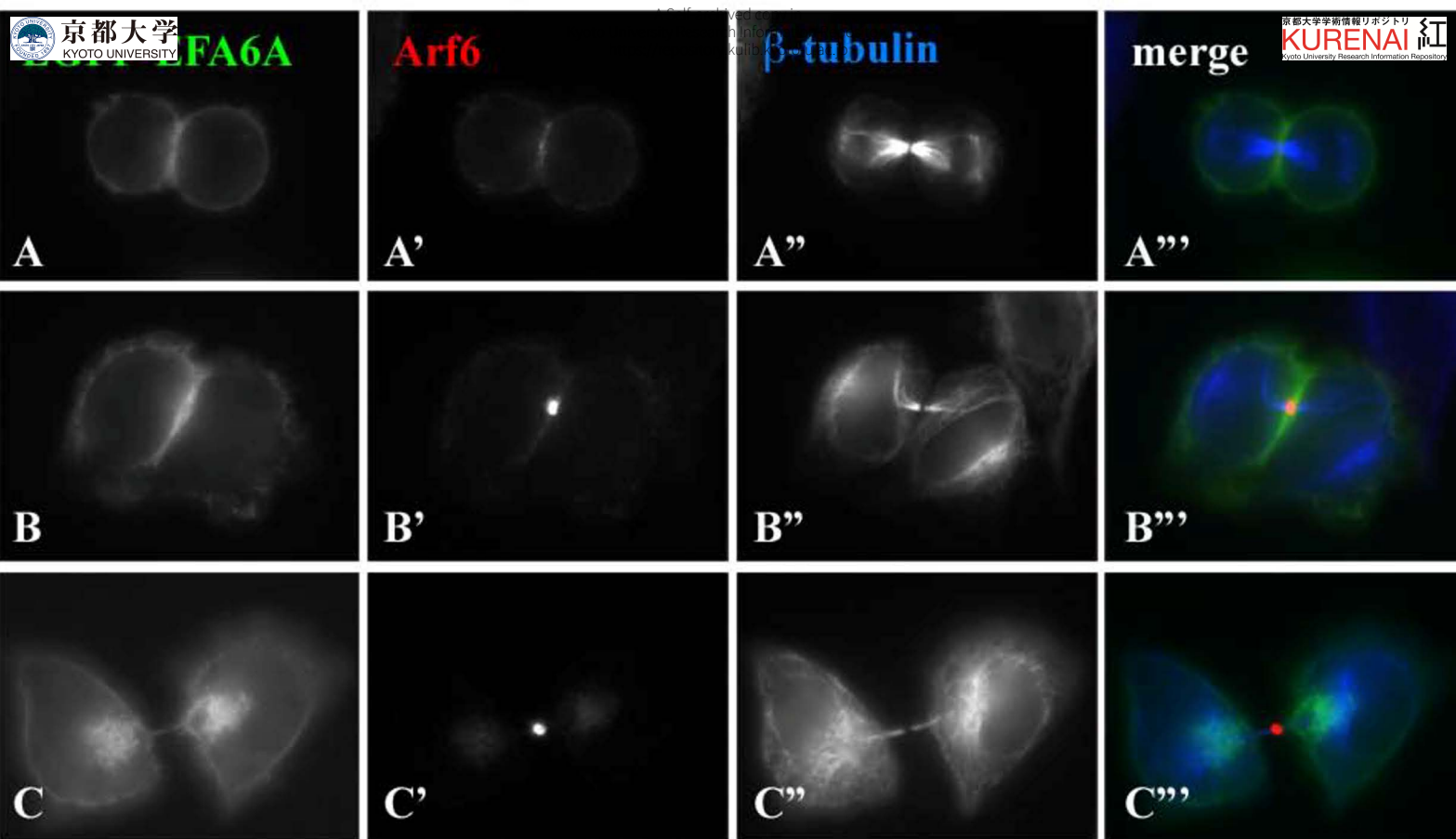


# EFA6A



**Fig. 2: Ueda et al.**



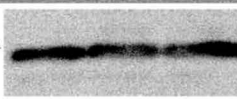


**Fig. 3: Ueda et al.**

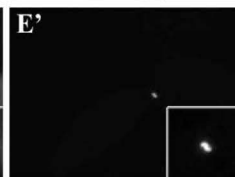
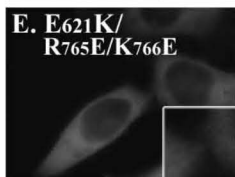
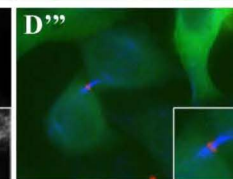
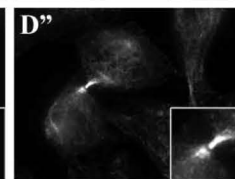
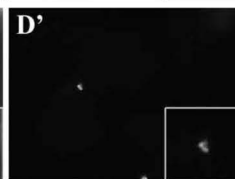
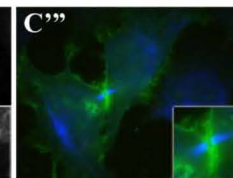
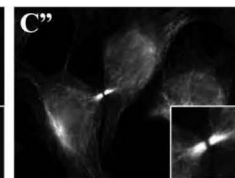
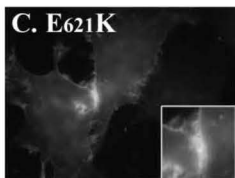
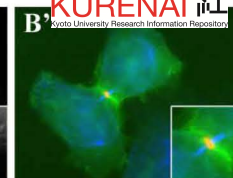
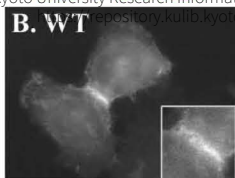
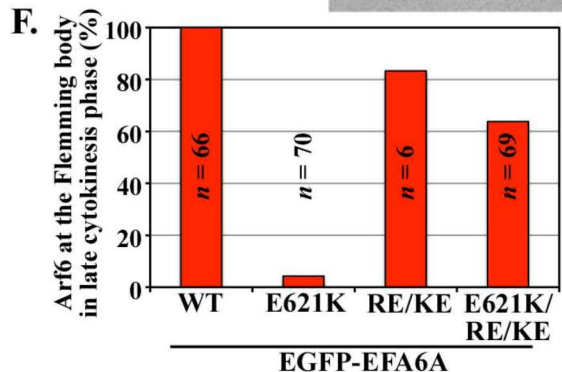
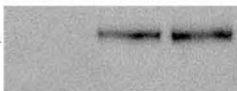
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(pulldown)



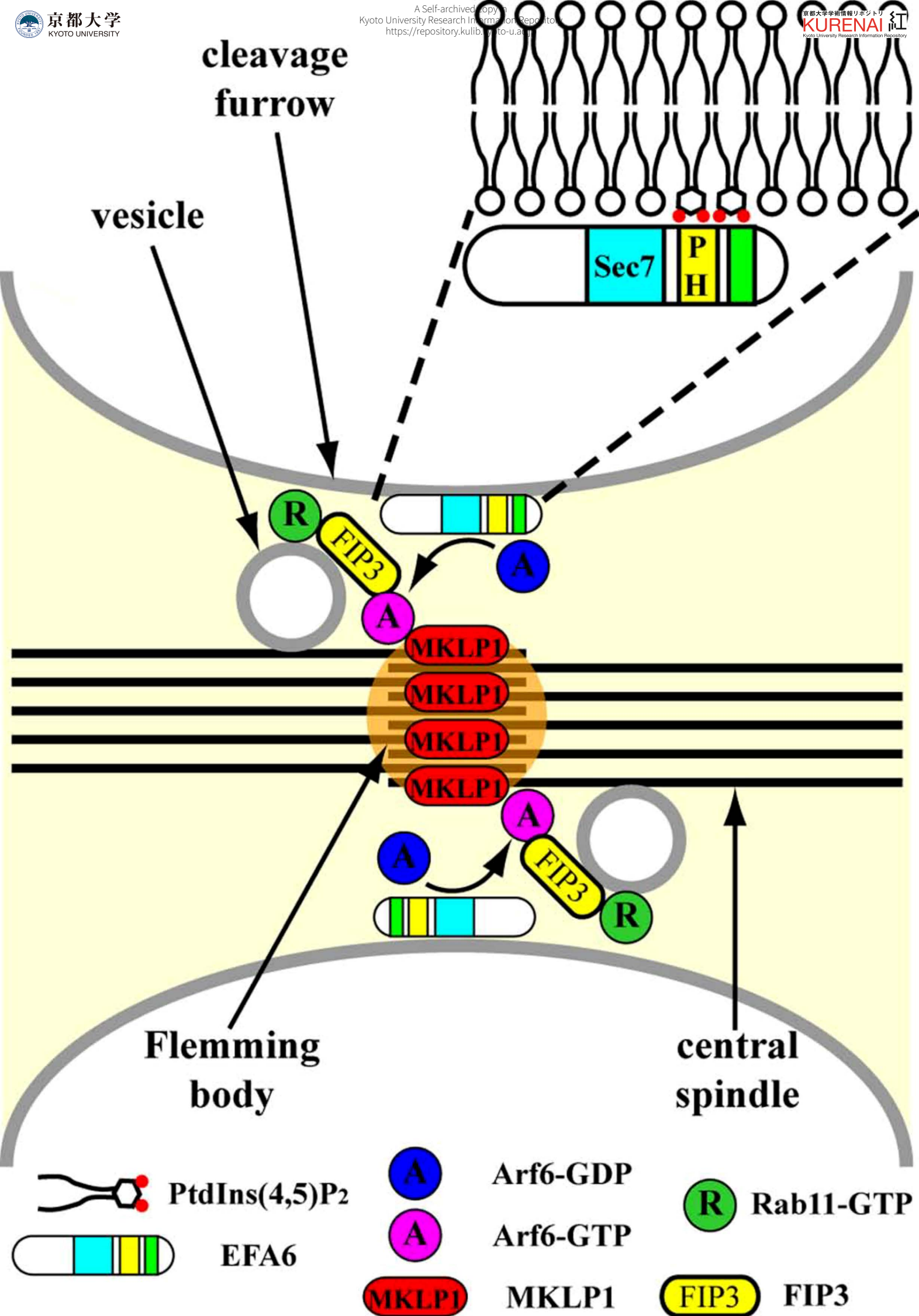
Arf6-HA  
(input)



EGFP-EFA6A  
(input)



**Fig. 4: Ueda et al.**



**Fig. 5: Ueda et al.**



## Supplementary Materials

### EFA6 activates Arf6 and participates in its targeting to the Flemming body during cytokinesis

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Senye Takahashi<sup>a</sup>, Hye-Won Shin<sup>a,c</sup>, and Kazuhisa Nakayama<sup>a,\*</sup>

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## Supplementary Figures

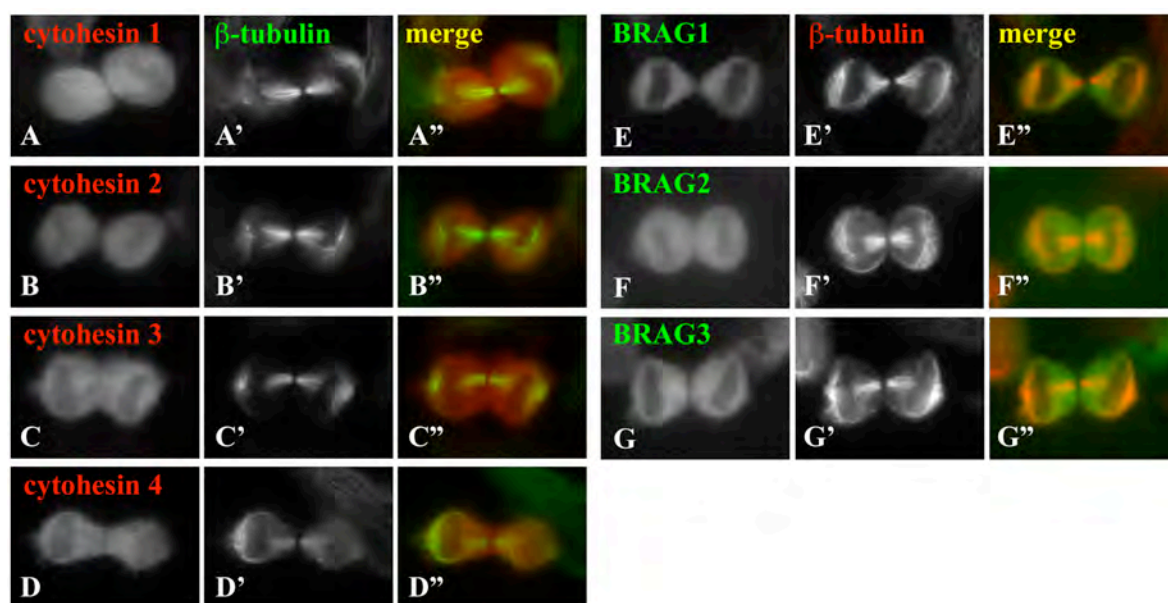


Fig. S1: Ueda et al.

### Fig. S1. Localization of cytohesin and BRAG isoforms during cytokinesis

HeLa cells transfected with an expression vector for N-terminally mCherry-tagged cytohesin 1 (A-A''), cytohesin 2 (B-B''), cytohesin 3 (C-C''), or cytohesin 4 (D-D''); or N-terminally EGFP-tagged BRAG1 (E-E''), BRAG2 (F-F''), or BRAG3 (G-G'') were processed for staining with anti-β-tubulin antibody (A'-G'). Representative images of cells in late cytokinesis phase are shown. None of the cytohesin or BRAG is localized to the cleavage furrow or on the Flemming body.

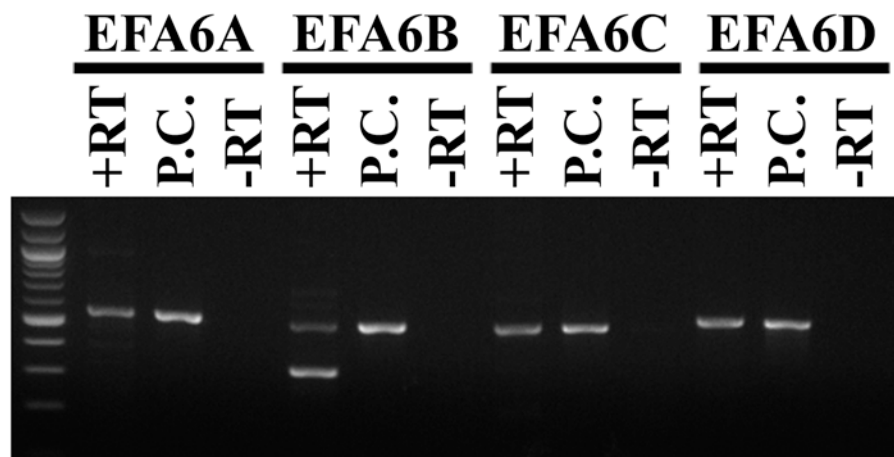


Fig. S2:Ueda et al.

### Fig. S2. Expression of EFA6 family members in HeLa cells

HeLa cell total RNA was transcribed with reverse transcriptase (+RT) or mock-transcribed without reverse transcriptase (-RT); cDNA was subjected to PCR using a set of primers specific for each EFA6 isoform (Table S1). As a positive control (P.C.), a plasmid vector containing each EFA6 cDNA was also subjected to PCR.

### Supplementary Table

Table S1. PCR primer sets for human EFA6 isoforms

EFA6	forward or reverse	sequence
EFA6A	forward	5'-GTCCGACTCAGACTCAGAGC-3'
	reverse	5'-AGCTTCTCATTCTTGATGGAGC-3'
EFA6B	forward	5'-GCGCGCATCAACTTGGCTG-3'
	reverse	5'-ATTTGCGGCCGCTCACAGCTGATTGCGGTTCC-3'
EFA6C	forward	5'-GCTGACATGGATGGCAAGAG-3'
	reverse	5'-TTCAACCGGTACTCCTCGGC-3'
EFA6D	forward	5'-GCCAGCGACTCCATCTACAC-3'
	reverse	5'-CCCTCATTTACCCCTTGCAG-3'

### Supplementary Video Legend

Video S1. Spatiotemporal changes in the localization of mCherry-EFA6A and Arf6-EGFP during mitosis

HeLa cells transfected with expression vectors for mCherry-EFA6A and Arf6-EGFP were subjected to time-lapse recording as described in Experimental Procedures. Images were collected every 5 min. Frame rate, 3 frames per sec.